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Comparing Morphometric, Biochemical and Visual Measurements of Macrovesicular Steatosis of Liver

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Abstract

The degree of macrovesicular steatosis is typically evaluated in liver biopsies by visual estimation, which is subject to intra- and inter-observer variations. Computer morphometry and biochemical measurement may provide more accurate results. Our aim was to develop a morphometry method and compare its results with visual and biochemical measurements. Twenty-six fresh frozen liver specimens were each divided into four aliquots. Three aliquots were processed biochemically to extract fat, and the fat content was defined as the weight percentage of fat. One aliquot was fixed in formalin, from which hematoxylin and eosin slides were made and reviewed by three pathologists to estimate fat content. Digital images of slides were analyzed by computer morphometry, which defined fat content as the percentage of area occupied by fat droplets. The results showed that individually, each method produced highly precise and reproducible measurements. Compared with each other, they showed very strong correlations (correlation coefficient $r=0.81-0.95$). The range of fat content in all 26 specimens was 2.2–15% by biochemical, 0.8–82.5% by visual, and 0.3–19.6% by morphometry method. Visual estimation appeared to have a systematic bias, giving results nearly 4-fold higher than other methods. This may be because visual estimation denotes the fraction of hepatocytes containing fat droplets, instead of the true fraction of fat. Strong correlations between different methods suggest that all three are valid methods for measuring steatosis. Computer morphometry is easy to implement and not affected by the bias seen in visual estimation. It may serve as a potential supplemental or alternative method.

Keywords

macrovesicular steatosis; morphometry; method comparison

Introduction

Macrovesicular steatosis is a common finding in liver biopsies. It is the pathological hallmark for nonalcoholic fatty liver disease (NAFLD), and is frequently seen in many other

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Disclosure/Conflict of Interest

The authors declare no conflict of interest.

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chronic liver diseases as well. It is estimated that NAFLD affects more than 30 million Americans with obesity or diabetes.(1) If left untreated, NAFLD can progress to nonalcoholic steatohepatitis (NASH) and cirrhosis, leading to significant morbidity and mortality.(1) Microvesicular steatosis is another rare histological subtype of steatosis that is often caused by acute drug toxicity leading to mitochondrial injury.(2) The current study limits its scope to macrovesicular steatosis.

The degree of macrovesicular steatosis is an important prognostic factor for NAFLD. More severe steatosis is associated with higher risk of disease progression to NASH.(3) In liver transplantation, a high degree of steatosis in donor livers has been linked to increased graft failure and short survival.(4,5) Because of its clinical significance, the degree of steatosis, or fat content, is routinely evaluated in liver biopsies. In standard practice, fat content is assessed visually and semi-quantitatively on H&E slides, and a percentage is given by the pathologist. Although the actual meaning of this percentage has rarely been discussed in the literature, it usually denotes the proportion of hepatocytes containing fat droplets.(3,6) A four-tier grading system is often used, wherein grade 0 is <5%, 1 is 5–33%, 2 is 33–66% and 3 is >66%. Previous studies have shown that visual estimation is subject to inter- and intra-observer variations.(7)

Computer morphometry has been proposed as a potential alternative method for quantifying liver fat content.(8,9) This method uses computer image analysis software to identify and measure the area occupied by optically clear fat droplets on digitally scanned H&E slides. In addition to its objectivity and high reproducibility, morphometry can produce results as a continuous variable, which may be desirable for research purposes.

An intrinsic limitation shared by both the visual and morphometric methods is that only two-dimensional materials can be examined, raising the question whether the measured material is truly representative. Biochemical method, which involves homogenization of specimen and direct extraction of fat, is an approach that can give a global measurement.(10) However, tissue has to be sacrificed during processing.

Non-invasive imaging studies such as ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI) have also been used to assess the degree of steatosis.(11) The precision and accuracy of these methods are generally low, and some important information, like the pattern of steatosis (centrilobular vs. periportal), cannot be acquired.(12) Therefore, tissue-based methods, particularly visual estimation on H&E slides, remain the clinical standard.

The aim of the current study is two-fold: 1, to establish and validate a computer aided morphometric method for measuring liver fat content; 2, to study the accuracy of and the strength of correlation between the three different methods, biochemical, visual and morphometric.

Materials and Methods

Sample selection

Fresh-frozen liver tissues of deceased donors (n=26) were obtained from the LTPADS (Liver Transplant Procurement and Distribution Service, University of Minnesota, Minneapolis, MN). Four aliquots of liver tissue, approximately 50mg each, were collected from each donor. One aliquot was submitted for routine H&E histology, while the other three were processed biochemically for fat extraction. Institutional Review Boards at the University of Chicago had reviewed and approved this study.

Biochemical fat content measurement

Total fat was extracted using low toxicity organic solvents according to a modified, previously described protocol.(13) Frozen liver tissues (~50 mg) were homogenized in 0.3 ml cold PBS using ceramic beads technology on a Precellys 24 homogenizer (Bioamerica Inc.). Homogenates were transferred into glass test tubes and mixed with 2 ml of hexane/isopropanol (3:2) solution. To minimize lipid oxidation, the tubes were gassed with nitrogen before being closed. Samples were then incubated on a shaker at room temperature for 1 hour. At the end of the incubation 0.6 ml of Na₂SO₄ (0.5M) was added to remove non-lipids. Samples were vortexed for 1 minute and incubated on a shaker at room temperature for 15 minutes, then centrifuged at 1000g for 10 minutes. The upper phase of each sample containing lipids dissolved in hexane was transferred to a clean, previously weighed glass tube. Samples were then dried with nitrogen gas. The percentage of total fat was determined by the weight difference between tubes with lipids and clean tubes normalized by the initial amount of tissue. The measurement was carried out in triplicates, using three aliquots of liver tissue from each donor. The entire process took approximately 3 hours.

Visual fat content estimation by histology

Fresh-frozen liver tissue was fixed in 10% formalin and then embedded in paraffin. Sections were cut at 4µm and stained with H&E. The slides were reviewed by three pathologists (M.L., S.X. and J.H.) independently. Each pathologist gave an estimated percentage of steatosis with 5% interval. The percentage designates the proportion of hepatocytes involved by macrovesicular steatosis. The evaluation was repeated 4 weeks later without referring to the prior results.

Computer aided morphometry

The same H&E slides from visual method were used for computer aided morphometry. Digital images were taken at 100× magnification, using an Olympus BX41 microscope and an AxioCam (Carl Zeiss Inc.) digital camera, attached to an Intel computer (2.4GHz, 2GB RAM) running the AxioVision LE image acquisition software (Carl Zeiss Inc.). The settings of digital camera, microscope and software were kept the same for all cases. On average, 7 digital images were taken for each case, covering >90% area of tissue section. Morphometry was performed using ImagePro 6.0 software (Media Cybernetics Inc.). Image segmentation was done with the “color cube” method, by manually picking a few distinct fat droplets, which served as reference. The software’s proprietary algorithm then automatically identified objects with similar optical characteristics. These objects were regarded as potential fat droplets. For each object, three basic parameters were measured: area, perimeter and roundness. Roundness (R) was calculated by the formula $(4 \times \pi \times \text{area}/\text{perimeter}^2)$, which equals to 1 for perfectly round objects, and decreases towards 0 for more irregular objects. Filters were set to exclude exceedingly large objects (area > 5000 pixels, or ~3800 µm²), and objects with exceedingly low roundness ($R < 0.35$), which typically represented optically clear artifacts (such as cracks), or sinusoidal and vascular spaces, instead of true fat droplets. Qualified objects were highlighted and the processed images were manually inspected to ensure quality. Fat content was calculated as the percentage of total surface area occupied by fat droplets. The morphometric measurement was repeated twice (J.S.). Morphometry took approximately 15 minutes for each case.

Statistical analysis

The reproducibility of biochemical method was evaluated by the coefficient of variation (CV) among triplicates. The reproducibility of visual estimation was assessed for both intra- and inter-observer variations, measured by intra-class correlation coefficient (ICC). For morphometry, the variation between two measurements was determined by CV as well as

ICC. In correlation analysis, different methods were compared with each other by the mean result of multiple measurements for each case. Both Pearson's correlation coefficient (r) and Spearman's rank correlation coefficient (r_s) were calculated, using EXCEL and SPSS.

Results

The mean fat contents determined by different methods are summarized in Table 1. The range of absolute results differed significantly between the visual estimation technique and the other two methods: 2.2%–15% for biochemical (mean 6.1%, median 4.7%), 0.8%–82.5% for visual (mean 24.2%, median 8.8%), and 0.3%–19.6% for morphometric measurement (mean 6.2%, median 3%).

In terms of reproducibility, the biochemical method was shown to be highly precise, and the average CV of triplicates was 7.3% (range 1.8%–15.9%). For visual estimation, the intra-observer variation was very small (ICC=0.96 for J.H.; ICC=0.97 for M.L.; ICC=0.97 for S.X.), and the inter-observer variation was also minimal (ICC=0.97). For morphometry, the average CV between two experiments was 5.2% (range 0.1%–16.6%), and the concordance was very strong (ICC=0.99). These results support that all three methods are highly reproducible and precise.

Correlation studies showed excellent correlation between the three methods, with Pearson's correlation coefficient (r) ranging from 0.81 to 0.95, and Spearman's rank correlation coefficient (r_s) ranging from 0.77 to 0.92 (Table 2). Linear regression plots were shown on Figure 1.

Discussion

The extent of macrovesicular steatosis is an important prognostic factor for NAFLD, and more severe steatosis is associated with higher risk of NASH.(3) The degree of steatosis in donor liver has been shown to predict graft outcome.(4) It is therefore critical to measure steatosis accurately in order to guide clinical decisions. Visual estimation of fat content on H&E slides is the current standard of care, while computer aided morphometry and biochemical methods have been proposed as potential alternatives. Each of the three methods has strengths and potential biases. The visual method is convenient and most practical, but as a subjective estimation it is prone to inter- and intra-observer variations.(14) Morphometry requires special hardware and software, and has the same limitation as the visual method because only two-dimensional materials can be examined. The biochemical method can provide a global assessment of fat content, but tissue has to be sacrificed during processing. It may not be suitable for routine clinical use, especially when tissue is limited and needs to be preserved for histological examination. Several previous studies have compared visual versus biochemical and visual versus morphometric method, and have generally reported good correlation.(8,12,15–18) However, our study is the first to compare all three methods in a single study using human liver tissue from deceased donors. Since there lacks a universally accepted “gold standard” for liver fat content measurement, this type of three-way comparison is critical because different methods could validate each other. As our results showed, very strong correlation was present between all three methods, supporting that they are all valid methods for measuring steatosis.

The biochemical method has a unique advantage in the sense that it can measure essentially all the fat in a processed specimen. In our study, it appeared to be highly precise, since the variation among the triplicates was very low (average CV=7.3%). Fat content measured by this method would include both fat droplets (in cytoplasmic vacuoles) and intrinsic fat such as lipid in cell membrane. Therefore, even biopsies without visible fat droplets would have

some baseline fat content. This was indeed observed in our study, and this baseline appeared to be around 2% (Figure 1).

Visual estimation is the current standard practice for measuring steatosis. It is done during routine histological evaluation of liver biopsies. In our study, the intra-observer variation between two evaluations was very low ($ICC=0.96-0.97$), and the inter-observer variation among three pathologists was also minimal ($ICC=0.97$). However, variation could increase if more examiners participated. The accuracy of visual estimation was further confirmed by its strong correlation with both biochemical and morphometric methods. It is therefore reasonable to predict that visual estimation will remain as the mainstay of practice.

Morphometry uses the same routine H&E slides, but has been shown to be more reproducible than visual estimation.⁽¹⁵⁾ The variation between two morphometric measurements was minimal in our study (average $CV=5.2\%$, $ICC=0.99$). Furthermore, the absolute results of morphometry were essentially same as those of biochemical method, suggesting there was minimal systematic bias with morphometry. From our experience, morphometry can be done with widely available hardware and software, and the entire process takes approximately 15 minutes for one case. These characteristics make morphometry an attractive alternative, or at least a potential supplement, to standard visual estimation.

Despite the strong correlation among all three methods, the absolute result (percentage of fat content) differed significantly. Visual estimation consistently gave higher percentages than the other two methods, with nearly 4-fold difference on average. This indicates that visual estimation is systematically biased, most likely because pathologists tend to estimate the fraction of hepatocytes containing fat droplets, whereas biochemical method and morphometry measure the fraction of fat itself (either by area or by weight). This finding is consistent with previous reports stating that the degree of steatosis determined by visual estimation was systematically greater than morphometry.^(8,12) Nonetheless, it is important to point out that this bias does not make visual estimation invalid, as long as the results from different methods are not interchanged. A grading system could be adjusted for each individual method to standardize the results.⁽⁸⁾

In conclusion, we have developed a computer aided morphometric method to measure the degree of steatosis, and compared it with visual estimation and biochemical method. All three methods showed very strong correlation, supporting that they are all adequately accurate and reliable. Visual estimation appeared to have a consistent systematic bias in terms of absolute results, most likely due to its unique target of measuring (fraction of hepatocytes as opposed to fraction of fat) compared to the other two methods. On the other hand, morphometry did not seem to have the bias and its results closely resembled those of biochemical method. These results suggest that morphometry may provide some additional value especially when the absolute fat content is desired, and it could serve as a supplemental or alternative method for routine visual estimation.

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Figure 1.
Linear regression plots between different methods

Table 1

Fat contents measured by different methods

Case ID	Fat Contents (%)		
	Biochemical *	Visual *	Morphometric *
1	2.2	0.8	0.5
2	2.4	1.7	0.4
3	2.9	0.8	0.3
4	3.2	6.7	1.7
5	3.3	5.0	0.5
6	3.4	2.5	0.5
7	3.9	2.5	0.4
8	4.0	13.3	4.3
9	4.3	0.8	0.4
10	4.4	5.0	0.8
11	4.5	4.2	0.4
12	4.6	3.3	0.7
13	4.6	2.5	0.4
14	4.7	18.3	9.2
15	5.1	10.8	5.2
16	5.2	5.8	0.4
17	5.5	14.2	4.3
18	6.2	69.2	18.0
19	6.8	33.3	7.9
20	7.8	77.5	11.5
21	9.2	38.3	11.3
22	9.2	75.0	16.4
23	11.0	40.0	14.7
24	12.2	75.8	19.0
25	13.6	40.0	12.1
26	15.0	82.5	19.6

* Represent the average percentage when there are multiple measurements. Cases sorted by fat content determined by biochemical method.

Table 2

Correlation between different methods

Comparison	Pearson's r	Spearman's r_s
Biochemical vs. visual	0.81	0.87
Biochemical vs. morphometric	0.85	0.77
Visual vs. morphometric	0.95	0.92